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Growth Rate of Cultured Human Fibroblasts Increased by Glucocorticoids

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Selected glucocorticoids have been demonstrated to increase the growth rate of human skin fibroblasts in culture, over a physiologically significant concentration range.

At the same concentrations and identical conditions, the glucocorticoid compounds tested inhibited the growth rate of mouse L-929 cells. We have discussed currently acceptable theories of glucocorticoid mechanism of action that permit this dichotomous effect, the main point being that inhibition can no longer be regarded as the only response of fibroblasts to glucocorticoids. Conclusions drawn from observations of cell cultures affected by addition of glucocorticoids must have considered the source of the cells, as response may vary with source and biologic state of the cells in culture.

Glucocorticoids, structured for topical use, when added to mouse L-929 cell cultures inhibit their proliferation [1–3]. This formed the basis for a parallel line assay that predicted glucocorticoid clinical anti-inflammatory activity better than rat granuloma and thymus involution assays [4,5]. The cells have been subsequently utilized for other studies of glucocorticoid receptors [1,6], and contributed to the development of a unitary protein synthesis theory for glucocorticoid mechanism of action [3]. The assay is still highly regarded as a means for determination of glucocorticoid potency [7,8] and its recognized inhibitory effect is often quoted to account for the striae, skin thinning and telangiectasia observed from clinical applications of topical glucocorticoids.

Doubts, however, have been expressed of the applicability of the assay to the human situation. Winter and Wilson [9] found that the mouse fibroblast assay correlates neither with the vasoconstriction assay, nor with the atrophic skin thinning effect on the epidermis of the pig, nor with the clinical topical activity for fluocinolone acetonide, triamcinolone acetonide, and betamethasone valerate (paradoxically the weakest by fibroblast assay) series.

Another consideration is the outdated theoretical basis of the mouse fibroblast assay. The originators of the assay described transformation of fibroblasts to a rounded shape, reduced metabolic activity and growth rate, inhibition of new collagen formation and dissolution of already formed collagen as direct effects of glucocorticoids [4]. This correlated with the view originally proposed by Albright [10] that all extrahepatic cells, regardless of their genetic history are subject to antianabolic inhibitory glucocorticoid influence [3]. However, a great deal of accumulating evidence shows that glucocorticoids can increase proliferation in cell cultures of diverse origin, among them human cell cultures [11,12]. In addition to glucocorticoid production of increased growth rates [13], increased formation of DNA [14], RNA [15], proteins and other macromolecules [12] as glucocorticoid effects have been confirmed.

Kirk and Mittwoch [16] have found that fluocinolone acetonide and hydrocortisone had a transient stimulatory effect on human fetal skin fibroblasts promoting an early entry and lengthening in the DNA synthesis (S) phase of the cells. Cell growth rate was slightly increased. There were no inhibitory effects noted.

These observations agree with the current concepts of primary glucocorticoid mechanism of action which is one of mRNA and protein synthesis stimulation [17,18].

The often quoted parallel [3,4,5,19–22] between the anti-inflammatory and atrophic effects of topical glucocorticoids, and their inhibition of mouse L-929 fibroblasts in rapid growth phases, may be fortuitous. It is perturbing to find such an authority on the fibroblast as Leonard Hayflick [23] questioning the relevance of biological data obtained from studies of abnormal cell lines such as 3T3, BHK 21 and mouse L cells "which resemble no *in vivo* cell type."

We addressed ourselves therefore to comparing the effect of hydrocortisone, triamcinolone acetonide, desonide, and beclomethasone dipropionate on mouse L-929 and human adult diploid fibroblasts closely following the original Berliner and Ruhmann [4] and Brotherton [5] assay conditions which used only the L-929 fibroblasts.

MATERIALS AND METHODS

Five cell lines were used: *mouse* fibroblast NCTC clone L-929, ATCC No. CCL-1 (Flow Laboratories, Inglewood, California); *human* skin fibroblast ATCC No. CRL-1104 BiRil, male, 29 yr (American Type Culture Collection, Rockville, Maryland); *human* skin fibroblasts, Peggy and Ruth, females, 25 and 29 yr; and *human* skin fibroblast, Bruce, male 29 yr (courtesy Dr. H. Stich, Cancer Research Centre, University of British Columbia, Vancouver, Canada). The cells were maintained as a monolayer on 100 × 20 mm tissue culture places in Dulbecco's Modified Eagle Medium (MEM) with L-glutamine (Gibco), containing 3.7 gm sodium bicarbonate/liter medium, 15% fetal calf serum (Flow), gentamicin (Gentamycin, Gibco) 40 μg/ml medium, and amphotericin B (Fungizone, Gibco), 1.25 μg/ml medium. In later experiments, gentamicin was replaced by penicillin and streptomycin (100 μg/ml). The cells were incubated at 37° in 5% CO_2, 95% air. All human cells used were less than 12th passage. The mouse cells have been maintained by Flow Laboratories for many years. There have been reports indicating that the metabolic activity of mouse cell culture lines may be decreasing with the passage of years [24].

Solutions in a propylene glycol vehicle were prepared from hydrocortisone (Glaxo, Toronto, Ontario), desonide (Dome, Toronto, Ontario), triamcinolone acetonide (Squibb, Montreal, Quebec) and beclomethasone dipropionate (Glaxo), using the method of Brotherton [5]. Control solutions using the propylene glycol vehicle alone were also prepared. Handling of steroid and control solutions was identical.

In the assay procedure, 50,000 mouse or 100,000 human fibroblasts were transferred in 5 ml of medium to a 60 × 15 mm tissue culture plate (Falcon) on Day 0. The medium was exchanged for a fresh one containing glucocorticoids on Day 1, allowing cells to adhere as well as to enter log growth phase. The cells were counted on Day 4.

The fibroblasts were harvested with the aid of trypsinization (0.25% trypsin without calcium and magnesium in Hank's BSS, Flow), scraping with a rubber policeman, vigorous pipetting, and counting on a Coulter Counter, Model B. The contact time of the trypsin solution with mouse

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fibroblast cells was 1 min, with human fibroblasts, 5 min. Cell separation by vigorous pipetting alone or with the aid of DNAse in place of trypsinization was tried, but in our hands was inferior. Brotherton [5] states trypsinization is contraindicated for L-929 cells. In our experience 1 minute's trypsinization for mouse cells and 5 min for human cells, together with careful scraping with a rubber policeman, gives the most reproducible results. Berliner used trypsinization.

RESULTS

All glucocorticoids tested demonstrated a clear inhibition of mouse L-929 fibroblasts. Equally clearly, they stimulated growth rates of human skin fibroblasts derived from 5 different cell lines under identical media and logarithmic growth rate conditions. For human fibroblasts (100,000 inoculum, 60-mm plates), cell population increase during the 4-day growth period reached between 200,000 to 400,000. For mouse fibroblasts (50,000 inoculum, 60-mm plates), cell population increase during the 4-day growth period reached between 500,000 to 1,-500,000. Such 2- to 3-fold variations have been experienced and exceeded by other workers [5,19]. Relative inhibition or stimulation therefore, is expressed in terms of percent of means of simultaneous controls ± the largest coefficient of variation encountered among repeat experiments (Table). The coefficient of variation is the standard deviation expressed as the percent of the mean cell counts observed in one run. (Two to 4 duplicate plates were used to test the effect of a selected glucocorticoid concentration in each experiment). Comparisons by *t*-test showed that cell population increases (or decreases) under the influence of glucocorticoids which were greater than 10% of controls were significant at the $P < 0.01$ or less. Therefore, cell population differences larger than 10% were considered large enough for the detection of inhibitory or stimulatory effect and are shown outside the control range (Fig 1).

Growth stimulation of human skin fibroblasts and growth inhibition of mouse cells for all glucocorticoids were concentration dependent as shown for triamcinolone in the Table. Figure 1 and the Table are representative of cell counts after 3 days of glucocorticoid addition (4 days after subculture). Trial experiments determining numbers of cells 4 and 10 days after glucocorticoid addition showed that both stimulation and inhibition were even more pronounced, although by Day 10, both mouse and human cells had grown to confluence.

Berliner and Ruhmann [4] demonstrated triamcinolone acetonide to be 156 times more potent than hydrocortisone from their effects on mouse L-929 cells. Our dose-response curves of these steroids correspond (Fig 2). The dose-response curves for desonide and beclomethasone dipropionate fall between these 2 reference steroids, indicating lower potency than triamcinolone acetonide, but greater than hydrocortisone, as expected from clinical data. For the purposes of this paper, however, we claim no exact determination of relative potency values.

The growth response of the cell populations changed rapidly with small increases in glucocorticoid concentration (Fig 1). Logarithmic number of cells vs glucocorticoid concentration plots became approximately linear over this limited concentration range, which started at a lower concentration for more potent glucocorticoids, in agreement with the previous investigations of mouse L-929 fibroblasts reported [4,5]. Figure 2

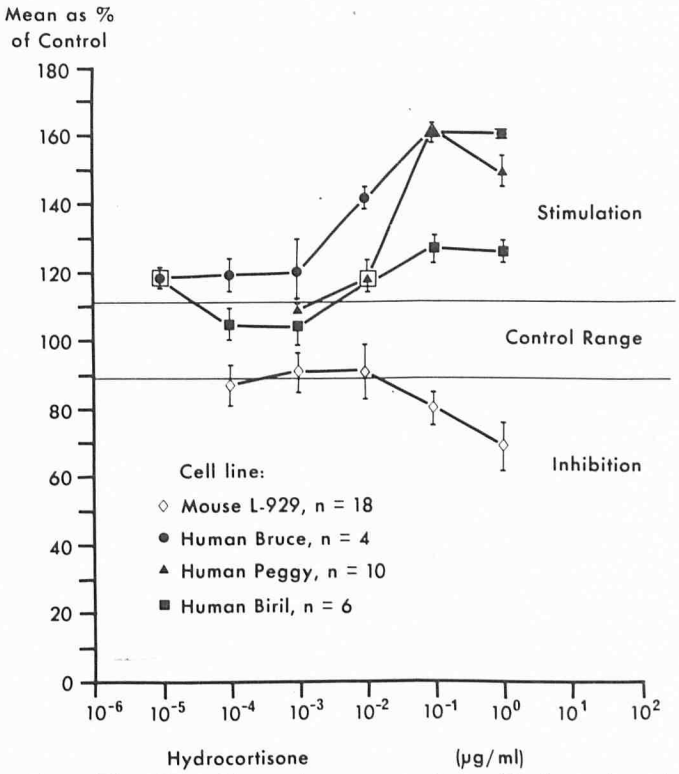

FIG 1. The effect of increasing concentrations of hydrocortisone in inhibiting growth of mouse L929 cells, and stimulating growth of 3 human cell lines.

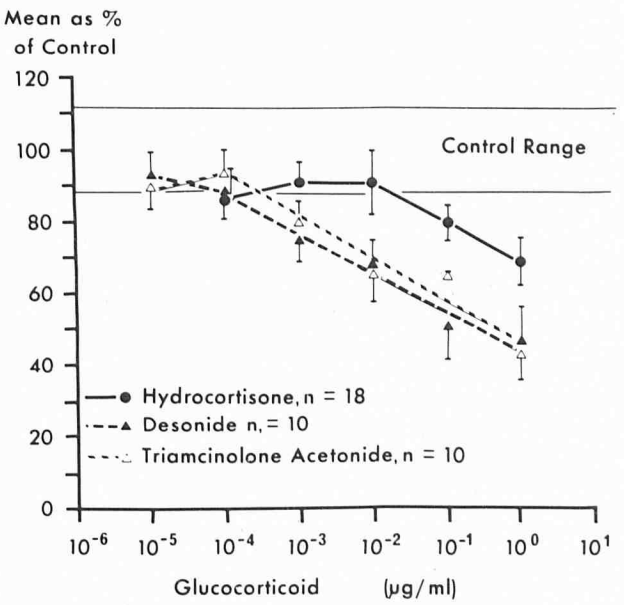

FIG 2. The effect of increasing concentrations of 3 different glucocorticoids in inhibiting the growth rate of mouse L929 cells.

Triamcinolone acetonide effect on mouse and human fibroblast growth

μg steroid/ml medium	Cell strain and number of observations (n)							
	Mouse L-929	(n)	Human Peggy	(n)	Human BiRil	(n)	Human Bruce	(n)
0.00001	93 ± 7[a]	(12)	—		122 ± 2	(4)	111 ± 4	(4)
0.0001	89 ± 6	(12)	—		121 ± 4	(4)	112 ± 5	(4)
0.001	75 ± 6	(10)	149 ± 1	(10)	125 ± 5	(6)	136 ± 5	(4)
0.01	68 ± 9	(6)	150 ± 5	(10)	124 ± 8	(4)	132 ± 4	(4)
0.1	51 ± 8	(10)	158 ± 3	(10)	130 ± 8	(6)	130 ± 4	(4)
1.0	47 ± 9	(4)	140 ± 4	(6)	113 ± 5	(4)	115 ± 6	(2)

[a] Mean as % of control ± largest coefficient of variation within series. Note: For brevity, the values for triamcinolone acetonide only are shown. Comparable values were obtained with hydrocortisone, desonide and beclomethasone dipropionate. (Fig 2).

graphically shows 38 observations of mouse cells 3 days after addition of 3 selected glucocorticoids. Beclomethasone dipropionate produced similar inhibition effects, but was not added to this figure to avoid a confusing clustering of observation points.

The topical steroids more potent than hydrocortisone by vasoconstriction assay produced more pronounced suppression at lower concentration than the reference glucocorticoid. This growth inhibitory effect continued up to the highest concentration investigated (10 μg/ml). At this concentration, hydrocortisone plates showed approximately 70% and the more active glucocorticoids 45% of the cell population of their respective controls.

Growth inhibition of mouse cells continued to increase with increasing glucocorticoid concentration, while human fibroblast stimulation reached a peak for all tested glucocorticoids at the level of 150–160% over controls. The peak concentration varied with glucocorticoid potency, stronger steroids reaching their peak effect as low as 0.001 μg/ml, the weaker hydrocortisone reaching it at higher concentrations of 0.1–1.0 μg/ml. The precise concentration of each steroid's maximum stimulatory effect was not sought. Human fibroblasts appeared by their growth response to discriminate less clearly between chemical structure differences than mouse fibroblasts did. The human fibroblast response in this respect correlates with the clinical experience which allows recognition of a change in concentration of the potent steroids only by a factor of 10–40 as compared with hydrocortisone (0.25%–0.01% for triamcinolone acetonide vs 0.5%–1.0% for hydrocortisone). For comparative purposes, it may be noted that the physiological hydrocortisone concentration in plasma is commonly regarded to be 0.04–0.2 μg/ml.

DISCUSSION

The results demonstrate that a physiologically significant concentration range exists in which all glucocorticoids tested are able to stimulate the growth rate of human skin fibroblasts in culture. Under the same conditions and glucocorticoid concentrations, mouse L-929 cell growth rates are inhibited.

Stimulation of human cell growth by glucocorticoids has again been observed previously [12,14,25], and in the case of 1 compound (hydroxymethylprednisolone) tested by Ruhmann and Berliner [21], growth stimulation of mouse L-929 cells occurred.

Berliner and Ruhmann [19] related the glucocorticoid inhibition of mouse L-929 cell growth rates to steroid anti-inflammatory activity and Berliner, Panagiotis, and Nabors [26] related the reduced growth rates to decreased collagen content. Atrophy and other adverse clinical steroid effects were attributed to this process and the concept of fibroblast inhibition as a cause of these undesirable effects has dominated clinical concepts for 25 yr.

Our experiments as described on glucocorticoid effect on fibroblasts have revealed the dichotomous effects of mouse L-929 cell growth inhibition and human fibroblast growth stimulation. It must be pointed out that most tissue culture work has been done on cultures in a stationary confluent phase with transformed cell lines which lack many of the responses to growth control effectors operative *in vivo*. This renders them poor models in the study of physiologically significant growth control mechanisms. This may be one of the reasons why the glucocorticoid growth rate stimulating effect on cells in log growth phase that we observed, has not yet been reported. Now that the techniques of growing human skin fibroblasts have become more widely available, we find confirming evidence in the work of other investigators [12]. Cunningham, Thrash, and Glynn [13] experimented with mouse 3T3, L-929, malignant Py3T3, malignant SV3T3 and human foreskin fibroblasts. They found that hydrocortisone stimulated both the growth rates and DNA synthesis of the 3T3 and human foreskin fibroblasts, but not the L-929 and the malignant cells. They considered that the following 3 points should be considered to account for the observed differences between the L-929 and the human fibroblast responses to glucocorticoids. (1) Stimulation by cortisol might require higher levels of serum than were employed in the experiments (10%) because stimulation might depend on prior binding of hydrocortisone to a serum protein such as transcortin. This can be hardly likely because we did use a higher serum concentration (15%) and triamcinolone acetonide does not bind to transcortin. (2) Hydrocortisone might initiate DNA synthesis only in the G_o phase of the cell cycle. An equivalent rest phase may not exist in such cultures in order to compare these unnatural cells with normal ones. (3) Intracellular cell components, e.g., cytosol glucocorticoid receptors may have been altered and become unresponsive. This is unlikely because they respond to glucocorticoids by growth inhibition.

Kirk and Mittwoch [16], in discussing their results, emphasized the biphasic nature of the cellular response to glucocorticoids and their role as homeostatic growth regulators possessing both enhancing and inhibitory properties.

The mechanisms which cause topical glucocorticoids to stimulate the growth rates of human skin fibroblasts but to inhibit mouse L-929 skin fibroblasts are unknown. It has been suggested by Tomkins that the glucocorticoid effect on L-929 or similar transformed cells may be due to a lack of a pleiotypic mediator in these cells, as appears to be the case in malignant cells [27]. According to Tomkins, all cells under unfavourable growth conditions such as absence of serum, are locked into a rest phase by a membrane-located negative regulator, the "pleiotypic mediator," probably a nucleotide. Transformed cell lines, such as the L-929's may lack this regulator. One effect of serum, glucocorticoids, or insulin, all of which stimulate cell proliferation, could be the displacement of "the pleiotypic mediator" from cell membrane. Displacement of this mediator is then followed by a series of metabolic stimulating effects, among them RNA synthesis, protein synthesis, polysome formation, nucleic acid precursor uptake and glucose uptake which would explain the observed growth rate stimulation. Such a stimulating effect, according to this theory, would be observed in early passage human skin fibroblasts, but not the L-929 cells.

The growth response shown by human skin fibroblasts to the administration of glucocorticoids thus may be a net result between glucocorticoid inhibitory activity and pleiotypic stimulatory activity. In the L-929 cells we would see only the inhibitory effect. This reasoning could explain the limited growth stimulation observed which was approximately the same for all glucocorticoids tested, and would also explain the unlimited inhibitory effect we observed on the L-929 cells. These cells continued to show increasing growth inhibition up to the highest concentrations used (10 μg/ml) for all glucocorticoids tested. No pleiotypic mediator has yet been identified, however.

The results of these experiments, as do those of Kirk and Mittwoch [16] indicate very clearly that it is unwise to accept without qualification, data obtained from studies that use heteroploid or genetically abnormal cell lines to evaluate potencies of topical glucocorticoids or to extrapolate those data to explain glucocorticoid effects in man. It is also apparent that generalizations of glucocorticoid mechanisms of action which assume a universal inhibitory effect on peripheral cells are misleading. Finally, as fibroblasts have many and varied functions, no generalization can be utilized to explain glucocorticoid effect on these cells.

Glucocorticoids were supplied by Dome Laboratories (desonide), Glaxo (beclomethasone dipropionate and hydrocortisone), and E. R. Squibb & Sons Ltd. (Triamcinolone acetonide).

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